

Comparison of immune responses in parenteral FaeG DNA primed pigs boosted orally with F4 protein or reimmunized with the DNA vaccine

V. Melkebeek^{a,*}, F. Verdonck^a, B.M. Goddeeris^{a,b}, E. Cox^a

^aLaboratory of Veterinary Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

^bDepartment of Biosystems, Faculty of Bioscience Engineering, K.U. Leuven, Kasteelpark Arenberg 30, B-3001 Heverlee, Belgium

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Abstract

We previously showed that an intradermal (i.d.) FaeG DNA prime (2×)-oral F4 protein boost immunization induces a systemic response and weakly primes a mucosal IgG response in pigs, especially when plasmid vectors encoding the A and B subunit of the *E. coli* thermo-labile enterotoxin (LT) are added to the DNA vaccine. In the present study, we evaluated whether addition of 1 α ,25-dihydroxyvitamin D₃ (vitD₃) to the DNA vaccine could further enhance this mucosal priming and/or modulate the antibody response towards IgA. To further clarify priming of systemic and mucosal responses by the i.d. DNA vaccination, we firstly compared the localization of the F4-specific antibody response in pigs that were orally boosted with F4 to that in pigs that received a third i.d. DNA immunization and secondly evaluated cytokine mRNA expression profiles after i.d. DNA vaccination. The i.d. DNA prime (2×)-oral F4 boost immunization as well as the 3 i.d. DNA vaccinations induced mainly a systemic response, with a higher response observed following the heterologous protocol. Co-administration of vitD₃, and especially of the LT vectors, enhanced this response. Furthermore, only the heterologous immunization resulted in a weak mucosal priming, which appeared to require the presence of the LT vectors or vitD₃ as adjuvants. In addition, the LT vectors strongly enhanced the FaeG-specific lymphocyte proliferation and this was accompanied by the absence of a clear IL-10 response. However, despite two DNA immunizations in the presence of these adjuvants and an oral F4 boost, we failed to demonstrate the secretory IgA response needed to be protective against enterotoxigenic *E. coli*.

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1. Introduction

Enterotoxigenic *E. coli* (ETEC) that express F4 (K88) fimbriae are an important cause of diarrhea in recently weaned piglets. The F4 fimbriae are long proteinaceous appendages mainly composed of several hundreds identical FaeG subunits. They enable the

bacteria to adhere to F4-specific receptors (F4R) on the intestinal epithelium and subsequently to colonize the small intestine (Nagy et al., 1985). Presence of the F4R is genetically determined and F4R negative (F4R[−]) pigs are resistant to an F4 positive (F4⁺) ETEC infection (Rutter et al., 1975). It was previously shown that oral immunization of F4R⁺ weaned pigs with F4 fimbriae resulted in an antigen-specific secretory IgA (sIgA) response at the intestinal mucosa, protecting these pigs against a subsequent F4⁺ ETEC challenge (Van den Broeck et al., 1999a). However, to prevent post-weaning

* Corresponding author. Tel.: +32 9264 73 84; fax: +32 9264 74 96.
E-mail address: Vesna.Melkebeek@UGent.be (V. Melkebeek).

diarrhea, an F4-specific intestinal mucosal immune response should already be primed during the suckling period, often in the presence of F4-specific maternal antibodies. These lactogenic antibodies will hamper the use of F4 as a vaccine. Several studies have shown that DNA vaccines, in contrast to conventional vaccines, can successfully prime immune responses in the presence of maternal antibodies (Hassett et al., 1997; Fischer et al., 2003; Van Loock et al., 2004). Therefore, we hypothesized that priming with an FaeG DNA vaccine during the suckling period combined with an oral F4 protein boost immediately after weaning could be an interesting approach. However, in a previous study a parenteral DNA prime-oral F4 boost induced a good systemic response, but was weak in priming mucosal immunity, failing to completely prevent F4⁺ *E. coli* colonization. Furthermore, addition of plasmid vectors encoding the LTA and LTB subunits to the FaeG DNA vaccine enhanced the IgG response, but did not result in the sIgA response that is desired to completely prevent an F4⁺ ETEC infection. Nevertheless, a significant reduction was obtained in the amount of F4⁺ ETEC excreted as well as in the duration of faecal F4⁺ ETEC excretion (Melkebeek et al., 2007). These data suggest that the systemic DNA immunization is weak in priming mucosal responses. Previous studies showed that a systemic immunization could induce an intestinal mucosal IgA response in mice (Enioutina et al., 1999, 2000) and in pigs (Van der Stede et al., 2001, 2004) if 1 α ,25-dihydroxyvitamin D₃ (vitD₃) was used as adjuvant.

Therefore, we examined in the present study whether addition of vitD₃ could enhance the mucosal priming by the FaeG DNA vaccination and/or whether it could modulate the antibody response towards IgA in the i.d. DNA prime-oral F4 boost immunization. Furthermore, to gain insight in the way systemic and mucosal responses are primed by the i.d. DNA vaccination, we compared the localization of the F4-specific antibody response in pigs that were orally boosted with F4 to that in pigs that were reimmunized i.d. with DNA and we evaluated cytokine mRNA expression profiles in the local draining lymph nodes after the i.d. DNA priming.

2. Materials and methods

2.1. Animals

Forty-nine conventionally bred pigs (Belgian Landrace x Piétrain), seronegative for antibodies against F4 as determined by ELISA, were weaned at the age of 4 weeks. Subsequently, they were housed in isolation

units. From 1 day before weaning, all animals were orally treated with colistine (150,000 U/kg body weight; Colivet, Prodivet Pharmaceuticals, Eynatten, Belgium) during 5 successive days to prevent *E. coli* infections during the weaning period.

2.2. Isolation of F4 fimbriae

F4 fimbriae were isolated from the ETEC strains GIS26, serotype 0149:K91:F4ac, LT⁺STa⁺STb⁺, and IMM01, serotype 0147:F4ac, LT⁺STb⁺ as described by Van den Broeck et al. (1999a). The protein concentrations of the fimbrial solutions were determined by the bicinchoninic acid reaction with bovine serum albumin as a standard (ICN Biomedicals, Belgium) and the purity was assessed using a Coomassie stained 15% SDS-PAGE and the Image Master 1D prime software (Amersham Pharmacia Biotech, Belgium). GIS26 F4 fimbriae were used for the oral immunization of pigs. The IMM01 strain carries F4 fimbriae with an FaeG sequence identical to the GIS26 strain, but fimbriae isolated from this strain have a higher purity, containing no flagellin (Verdonck et al., 2004). Therefore, IMM01 fimbriae were used for the FaeG-specific ELISA and ELIsot assays and, after sterilization by filtration through a 0.2 μ m filter, in an FaeG-specific lymphocyte proliferation assay.

2.3. Plasmids and 1 α ,25(OH)₂D₃

The pcDNA3-rpGM-CSF plasmid consists of the cDNA encoding the porcine GM-CSF cloned in the pcDNA3.1zeo expression vector.

The pWRGFaeGopt vaccine was constructed as previously described (Melkebeek et al., 2007). This construct consists of a codon optimized *faeG* cloned into the pWRG7079 vector behind a tPA signal sequence, allowing the extracellular secretion of the encoded FaeG. The pJV2004 and pJV2005 plasmids consist of the pWRG7054 vector encoding the A and B subunit of the thermolabile enterotoxin of *E. coli* (LT), respectively, behind a tPA signal sequence to allow their extracellular secretion (Arrington et al., 2002). All plasmids were propagated in *E. coli* DH5 α and large-scale purification of the plasmids was conducted by Qiagen Endofree plasmid kits (Qiagen GmbH, Germany). After determining purity and concentration by measuring the O.D. at 260 and 280 nm, the plasmids were stored at –20 °C.

1 α ,25(OH)₂D₃ (vitD₃) (Sigma), was dissolved in absolute ethanol at a concentration of 200 μ g/ml and stored at 4 °C.

2.4. Experimental procedure

2.4.1. Experiment 1

A first experiment was performed to confirm the induction of an F4-specific systemic immune response by i.d. immunization with pWRGFaeGopt or pWRGFaeGopt in combination with the LT vectors as well as the priming of an F4-specific intestinal mucosal IgG response by i.d. immunization with these vectors followed by an oral F4 boost as seen in a previous study (Melkebeek et al., 2007). Furthermore, we wanted to evaluate if the immunomodulating adjuvant vitD₃ could enhance the mucosal priming by the FaeG DNA vaccine and/or modulate the induced response towards an IgA response.

Hereto, 23 pigs were used (Table 1). One week post-weaning, they were divided into 4 groups and were all injected intradermally (i.d.) at the left site of the neck with 250 µg of pcDNA3-rpGM-CSF. Seven and 28 days later (days 0 and 21 post primary immunization (ppi)), the pigs were i.d. immunized at this site (at the pcDNA3-rpGM-CSF injection site) with 500 µg of pWRGFaeGopt (*n* = 7, pWRGFaeGopt group), pWRGFaeGopt supplemented with 100 µg pJV2004 and 100 µg pJV2005 (collectively referred to as the LT vectors) (*n* = 7, pWRGFaeGopt + LT group), pWRGFaeGopt supplemented with 5 µg of 1α,25(OH)₂D₃ (*n* = 5, pWRGFaeGopt + vitD₃ group) or the empty pWRG7079 vector (*n* = 4, pWRG7079 group). At day 42 ppi, 4 pigs of both the pWRGFaeGopt and the pWRGFaeGopt + LT group and 2 pigs of both the pWRGFaeGopt + vitD₃ and the pWRG7079 group received an identical i.d. booster immunization, whereas the remaining pigs (3 pigs of each immunized group and 2 pigs of the pWRG7079 group) were orally boosted with 1 mg F4 fimbriae diluted in 10 ml PBS during 3 successive days (days 42, 43 and 44 ppi, respectively).

The animals were deprived of food and water from 3 h before till 2 h after the oral immunization. The F4-specific humoral immune response was analyzed weekly by determining the F4-specific IgG, IgA and IgM titer in the serum of all pigs starting from day 7 till day 48 ppi. In order to localize and quantify the antibody response, the pigs were euthanized 6 or 7 days after the third immunization (day 48 or 49 ppi, respectively) for enumeration of the F4-specific IgG, IgA and IgM antibody-secreting cells (ASC) in the lymph node draining the DNA vaccination site (cervical superficial dorsal lymph node (Cerv LN)), the mesenteric LN (Mes LN), the ileal and jejunal Peyer's patches (IPP and JPP), the jejunal lamina propria (LP) and the spleen. To evaluate the induction of an F4-specific cellular response, an F4-specific lymphocyte proliferation assay was performed on spleen mononuclear cells (MC). Since the F4 receptor (F4R) is essential for inducing an intestinal IgA response, the presence of the F4R was determined on small intestinal villi as earlier described.

2.4.2. Experiment 2

A second experiment was performed to examine the induction of cellular immune responses (FaeG-specific lymphocyte proliferation and cytokine mRNA expression) in the local draining lymph node (Cerv LN) following priming with the FaeG DNA vaccine and to evaluate the influence of the intradermal LT vectors or vitD₃ on these cellular responses. Hereto, 26 pigs were used (Table 2). One week after weaning, 6 pigs were i.d. injected in the neck with sterile PBS (PBS group) and the other pigs with 250 µg of pcDNA3-rpGM-CSF. Seven and 28 days later (days 0 and 21 ppi), the pigs were i.d. injected at the same site with PBS (pigs from the PBS group) or 500 µg of pWRGFaeGopt (*n* = 5, pWRGFaeGopt group), pWRGFaeGopt supplemented

Table 1
Experimental design of experiment 1

Group	Day -7	DNA vaccine (days 0 and 21 ppi)	Third immunization (day 42 ppi)	Total number of pigs	F4R ⁺ pigs	F4R ⁻ pigs
pWRG7079 (control)	pcDNA3-rpGM-CSF	pWRG7079	pWRG7079	2	1	1
			Oral F4	2	1	1
pWRGFaeGopt (FaeG)	pcDNA3-rpGM-CSF	pWRGFaeGopt	pWRGFaeGopt	4	3	1
			Oral F4	3	1	2
pWRGFaeGopt + LT (LT)	pcDNA3-rpGM-CSF	pWRGFaeGopt + LT	pWRGFaeGopt + LT	4	3	1
			Oral F4	3	1	2
pWRGFaeGopt + vitD ₃ (vitD ₃)	pcDNA3-rpGM-CSF	pWRGFaeGopt + vitD ₃	pWRGFaeGopt + vitD ₃	2	1	1
			Oral F4	3	2	1

Table 2
Experimental design of experiment 2

Group	Total number of pigs	Day -7	Days 0 and 21 ppi	Number of pigs at 24 h psi	Number of pigs at 48 h psi
PBS	6	PBS	PBS	3	3
pWRG7079	4	pcDNA3-rpGM-CSF	pWRG7079	2	2
pWRGFaeGopt	5	pcDNA3-rpGM-CSF	pWRGFaeGopt	3	2
pWRGFaeGopt + LT	6	pcDNA3-rpGM-CSF	pWRGFaeGopt + LT	2	4
pWRGFaeGopt + vitD3	5	pcDNA3-rpGM-CSF	pWRGFaeGopt + vitD3	2	3

with 100 µg of each LT vector ($n = 6$, pWRGFaeGopt + LT group), pWRGFaeGopt supplemented with 5 µg of $1\alpha,25(\text{OH})_2\text{D}_3$ ($n = 5$, pWRGFaeGopt + vitD₃ group) or pWRG7079 ($n = 4$, pWRG7079 group). The pigs were euthanized 24 or 48 h after the second immunization (psi) as shown in Table 2 and for each pig the cervical superficial dorsal LN was collected at the left site (Cerv LN) and the right site (control Cerv LN) of the neck to analyze cytokine expression profiles. Cerv LN MC isolated at 48 h psi were also used in an F4-specific lymphocyte proliferation assay.

In both experiments, plasmids were diluted in 1 ml sterile PBS and both the i.d. injection with pcDNA3-rpGM-CSF as the i.d. immunizations were performed by multiple injections. Euthanasia of all pigs was performed by intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) followed by exsanguinations.

2.5. Spleen, Cerv LN, Mes LN, JPP, IPP and LP MC

MC were isolated from the spleen, Cerv LN, Mes LN (jejunal and ileal), JPP, IPP and LP as described by Verdonck et al. (2002) and finally resuspended in leukocyte medium [RPMI-1640 (GIBCO BRL) supplemented with penicillin (100 IU/ml) (GIBCO BRL), streptomycin (100 µg/ml) (GIBCO BRL), kanamycin (100 µg/ml) (GIBCO BRL), L-glutamin (200 mM) (GIBCO BRL), sodiumpyruvate (100mM) (GIBCO BRL), non-essential aminoacids (100 mM) (GIBCO BRL), 2-mercaptoethanol (5×10^{-5} M) (GIBCO BRL) and either 5% serum of a colostrum-deprived pig for the lymphocyte proliferation assay or 10% FCS for the ELISPOT assay]. Cerv LN MC were also resuspended in TRIzol Reagent (Invitrogen, Merelbeke, Belgium) (Cerv LN) to analyze cytokine mRNA expression.

2.6. RNA extraction and RT-PCR

RNA was extracted from 10^7 MC/sample using TRIzol Reagent following the manufacturer's protocol.

Reverse transcription was performed as described by Verfaillie et al. (2005). The oligonucleotide primers described by Verfaillie et al. (2005) were used for the detection of the porcine cytokines IL-2, IL-4, IL-6, IL-10, TGF-β, IFN-γ, TNF-α and of cyclophilin cDNA. Oligonucleotide primers used for the detection of β-actin and GAPDH cDNA were designed from the published nucleic acid sequences available from the GenBank/EMBL databases using the primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 3).

Real-time PCR reactions were carried with the LightCycler and the LightCycler-faststart DNA Master SYBR Green I kit (Roche, Mannheim, Germany). The reactions for IL-2, IL-4, IL-6, IL-10, TGF-β, IFN-γ, TNF-α and of cyclophilin were performed as described by Verfaillie et al. (2005). For β-actin and GAPDH, the reaction mixture was made following the manufacturer's protocol using 3 mM MgCl₂. After heating the samples at 95°C for 10 min, the temperature cycling consisted of denaturation at 94° for 15 s, annealing at 53°C (GAPDH) or 60 °C (β-actin) for 5 s, and extension at 72°C for 14 s. For β-actin 35 cycles and for GAPDH 40 cycles were performed. Fluorescence acquisition was measured at 85 °C in single mode. Melting curve analysis was done at 65–98 °C with continuous fluorescence acquisition. As an additional control of specificity, PCR products were subjected to agarose gel electrophoresis. Quantification occurred using external standards of cDNA and the copy number of each sample transcript was determined with the aid of the LightCycler software. Cyclophilin, GAPDH and β-actin were used as constitutively expressed reference genes and a normalization factor (NF) was calculated as the geometric mean of the copy numbers of these 3 genes to determine the uniformity of the reverse transcription and as a reference for quantification of cytokine mRNA. The relative amounts of cytokine-expression were calculated as a ratio of the copy number of the target cytokine over the NF and results are presented as the ratio of the relative amount of the target cytokine in the immunized Cerv LN over the relative amount of the

Table 3
Oligonucleotide primers and fragment length of PCR products for different porcine cytokines

Cytokine	Primers (5'-3')	Length PCR fragment	Genbank accession number
IL-1 α	(S) TGCCAGCTATGAGCCACTTCC (AS) TGACGGGTCTCGAATGATGCT	336	X52731
IL-2	(S) GATTACAGTTGCTTTTGAAG (AS) GTTGAGTAGATGCTTTGACA	338	X56750
IL-4	(S) TACCAGCAACTTCGTCCA (AS) ATCGTCTTTAGCCTTTCCAA	311	F68330
IL-6	(S) ATGAGAATCACCACCGGTCTTG (AS) TGCCCCAGCTACATTATCCGA	310	M86722
IL-10	(S) CCATGCCCAGCTCAGCACTG (AS) CCCATCACTCTCTGCCTTCGG	295	L20001
IFN- γ	(S) ATGTACCTAATGGTGGACCTC (AS) CTCTCTGGCCTTGGAACATAG	360	X53085
TGF- β	(S) GACCCGCAGAGAGGCTATAG (AS) GAGCCAGGACCTTGCTGTAC	399	Y00111
TNF- α	(S) CAAGGACTCAGATCATCGTCTCA (AS) CATACCCACTCTGCCATTGGA	100	X54859
Cyclophilin	(S) TAACCCACCGTCTTCTT (AS) TGCCATCCAACCACTCAG	368	F14571
β -Actin	(S) ACGTGGACATCAGGAAGGAC (AS) ACATCTGCTGGAAGGTGGAC	210	U07786
GAPDH	(S) GGGCATGAACCATGAGAAGT (AS) AAGCAGGGATGATGTTCTGG	230	AF017079

(S): Sense; (AS): Antisense.

target cytokine in the control Cerv LN = (copy number of target cytokine in the Cerv LN/NF in the Cerv LN)/(copy number of target cytokine in the control Cerv LN/NF in the control Cerv LN).

2.7. FaeG-specific lymphocyte proliferation assay

To determine the FaeG-specific lymphocyte proliferation, a proliferation assay as described by Van der Stede et al. (2003) was performed on spleen or Cerv LN MC by adding F4 fimbriae (final concentration of 15 μ g/ml), medium (negative control) or concanavalin A (ConA, final concentration of 10 μ g/ml, positive control) to the wells of a 96-well plate (Cellstar, Greiner bio-one, Wommel, Belgium) containing 5×10^5 cells/well (final volume of 200 μ l). After 72 h at 37 °C in 5% CO₂, cells were pulse-labelled with ³H-thymidine (1 μ Ci/well) (Amersham ICN, Bucks, UK) and 18 h later, they were harvested onto glass fibre filters. The radioactivity incorporated into the DNA was measured with a β -scintillation counter (Perkin-Elmer, Life Science, Brussels, Belgium).

2.8. ELISA for FaeG-specific serum IgG, IgA and IgM antibodies

FaeG-specific titers were determined by an indirect ELISA on serum sampled as described by Van den Broeck et al. (1999b). Hereto, the wells of a 96-well microtitre plate (NUNC, Polysorb Immuno Plates, Roskilde, Denmark) were coated with IMM01 F4 fimbriae at a concentration of 5 μ g/ml in PBS. After 2 h incubation at 37 °C, the remaining binding sites were blocked overnight at 4 °C with PBS supplemented with 0.2% Tween[®] 80 (Merck Eurolab, Leuven, Belgium). Subsequently, the sera were added for 1 h at 37 °C in series of two-fold dilutions in ELISA dilution buffer (PBS, pH 7.4 + 0.2% Tween[®] 20 + 3% BSA), starting at a dilution of 1/10. Then, optimal dilutions of anti-swine IgM-, IgA- or IgG-specific MAb (28.4.1, 27.8.1 and 23.3.1b, respectively, Van Zaane and Hulst, 1987) in ELISA dilution buffer were added to the wells for 1 h at 37 °C. Thereafter, rabbit anti-mouse HRP-conjugated serum (Dako, Denmark) optimally diluted in ELISA dilution buffer and supplemented with 2% pig serum was added for 1 h at 37 °C. Finally, ABTS (Roche

Diagnostics, Brussels, Belgium), containing H_2O_2 , was added and after 30 min incubation at 37°C , the optical density was measured spectrophotometrically at 405 nm (O.D._{405}). Between each incubation step, the plates were washed three times with ELISA washing buffer (PBS, pH 7.4 + 2% Tween 20). Cut-off values were calculated as the mean O.D._{405} of all sera (dilution 1/10) on day 0 ppi increased with three times the standard deviation and were 0.16, 0.23 and 0.63 for IgA, IgG and IgM, respectively. The antibody titer was the inverse of the highest dilution which still had an O.D._{405} higher than the calculated cut-off values.

2.9. *Elispot assay for FaeG-specific IgG, IgA and IgM ASC*

F4-coated plates were prepared as described for the ELISA. Thereafter, MC suspensions at a concentration of 10^7 cells/ml were added ($100\ \mu\text{l}$ /well) and plates were incubated for 14 h at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, the cells were removed by six washes with ELISA washing buffer whereafter wells were sequentially incubated for 1 h at 37°C with the anti-swine IgM-, IgA- or IgG-specific MAb and with a rabbit anti-mouse HRP-conjugated serum. Between these steps, the plates were washed 3 times with ELISA washing buffer. Subsequently, a substrate solution, consisting of 4 volumes of 3-amino-9-ethylcarbazole (AEC) (Sigma) working solution (0.67 ml AEC stock solution (0.4% in dimethylformamide) in 10 ml Na acetate (0.1 M, pH 5.2) + $10\ \mu\text{l}$ 30% H_2O_2) and 1 volume of 3% low-melting-point agarose (BIOzym, Landgraaf, The Netherlands) was added. After overnight incubation in the dark at room temperature, spots were counted with an inverted microscope. For each MC suspension, spots in five wells (10^6 MC/well) were counted to obtain the number of isotype-specific ASC/ 5×10^6 MC. Results are presented as the mean number of ASC per 5×10^6 MC \pm S.E.M.

2.10. *Statistical analysis*

Statistical analysis was done using SPSS 12.0 for Windows. Analysis of differences between the groups in \log_2 serum antibody titers was done using General Linear Model (Repeated Measures Analysis of Variance) using the Bonferoni adjustment for multiple comparisons. Differences between groups in lymphocyte proliferation and in relative increase in cytokine expression at the immunization site were tested for statistical significance with the Kruskal–Wallis Test. $P \leq 0.05$ was considered as statistically significant.

3. Results

3.1. *Experiment 1: FaeG-specific antibody responses following DNA vaccination*

3.1.1. *F4R characterization of the pigs*

As shown in Table 1, the in vitro villous adhesion assay demonstrated that 6 of the 11 pigs that were orally boosted with F4 and 4 of the 12 pigs that were i.d. boosted with DNA lacked the F4R.

3.1.2. *FaeG-specific serum antibody response following 2 intradermal DNA vaccinations*

Pigs were immunized i.d. with DNA at days 0 and 21 ppi. Since pre-injection with pcDNA3-rpGM-CSF enhances both humoral and cellular responses in pigs (Melkebeek et al., 2006), all pigs received an i.d. injection with this plasmid seven days before the first immunization (Table 1).

FaeG-specific serum IgG was already observed after 1 immunization with pWRGFaeGopt and addition of LT enhanced this IgG response (Fig. 1). VitD₃ was a less powerful adjuvant, enhancing the IgG response to a lesser extend. Control animals vaccinated with pWRG7079, did not show an IgG response. Consequently, the mean FaeG-specific IgG titer was significantly higher in the pWRGFaeGopt + LT than in the pWRGFaeGopt and the pWRG7079 group and in the pWRGFaeGopt + vitD₃ group than in the pWRG7079 group from 7 days after the second immunization (day 28 ppi) till the third immunization (day 42 ppi).

Levels of FaeG-specific IgA and IgM antibodies remained very low (mean titers ≤ 18 in all groups).

3.1.3. *FaeG-specific responses following an oral F4 boost or a third i.d. DNA vaccination*

To determine if the i.d. DNA vaccination induced an FaeG-specific intestinal mucosal antibody response or primed the gut-associated lymphoid tissues (GALT), pigs were either immunized a third time with DNA (day 42 ppi) or obtained an oral boost with 1 mg F4 fimbriae during 3 successive days (days 42, 43 and 44 ppi) (Table 1). Mucosal and systemic responses were analyzed 6 or 7 days later.

3.1.3.1. *The FaeG-specific serum antibody response.* Both the oral F4 boost and the i.d. DNA vaccination further enhanced the FaeG-specific serum IgG titers in all the FaeG DNA primed groups, irrespective of the F4R status of the animals, whereas the pWRG7079 animals remained negative (Fig. 2).

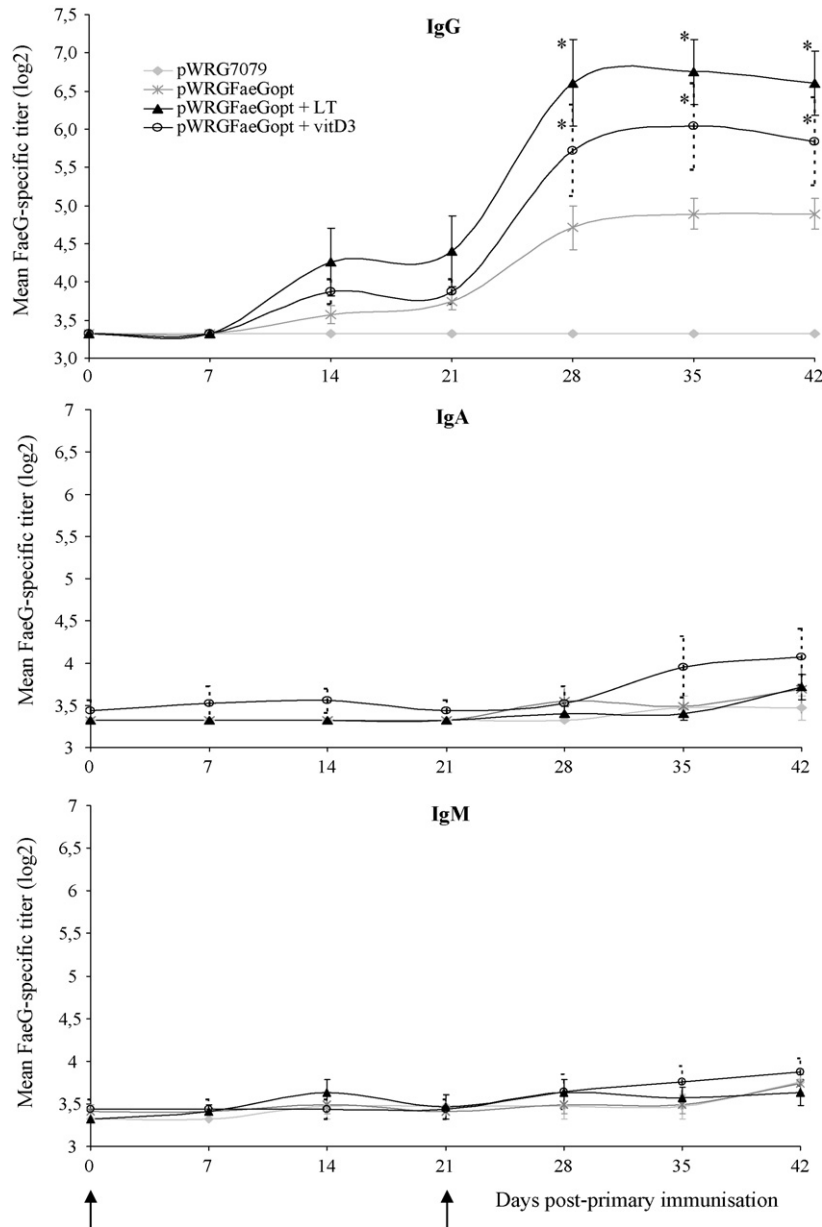


Fig. 1. FaeG-specific serum IgG, IgA and IgM titers following 2 i.d. immunizations with pWRGFaeGopt ($n = 7$), pWRGFaeGopt + LT ($n = 7$), pWRGFaeGopt + vitD3 ($n = 5$) or pWRG7079 (controls, $n = 4$) (days 0 and 21 ppi, black arrows). Significant differences ($P < 0.05$) between the pWRGFaeGopt + LT group and the pWRGFaeGopt and the control group and between the pWRGFaeGopt + vitD₃ group and the control group are indicated with the symbol (*). Results are presented as means \pm S.E.M.

None of the groups showed increased FaeG-specific serum IgA or IgM titers following the DNA or the F4 boost (data not shown).

3.1.3.2. Localization of the FaeG-specific antibody response. FaeG-specific ASC were mainly detected in the Cerv LN (Fig. 3) and to a lesser extend in the spleen (Fig. 4). In the control group, no FaeG-specific ASC

were identified in the Cerv LN, whereas a background of FaeG-specific IgM was identified in the spleen after the third injection with pWRG7079 (negative controls, $16\text{--}21 \text{ ASC}/5 \times 10^6 \text{ MC}$) as well as after the oral F4 immunization ($18\text{--}44 \text{ ASC}/5 \times 10^6 \text{ MC}$).

In all FaeG DNA vaccinated groups, the third i.d. DNA vaccination induced mainly FaeG-specific IgG ($7\text{--}305 \text{ ASC}/5 \times 10^6 \text{ MC}$) and to a lesser extend IgM

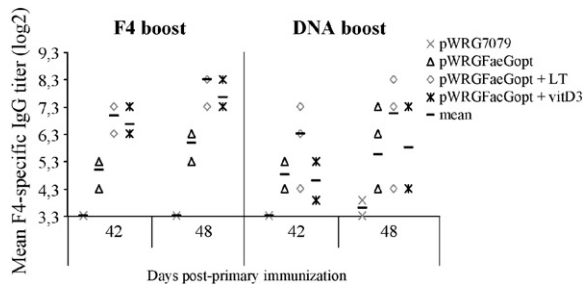


Fig. 2. Mean FaeG-specific serum IgG titer at the day of the third immunization (day 42 ppi) and 6 days later (day 48 ppi). Pigs were immunized as shown in Table 1.

ASC (3–92 ASC/5 × 10⁶ MC) in the Cerv LN, whereas mainly IgM (15–209 ASC/5 × 10⁶ MC) and to a lesser extend IgG ASC (0–39 ASC/5 × 10⁶ MC) were induced in the spleen. Moreover, the numbers of FaeG-specific ASC in the Cerv LN and the spleen were comparable for the three FaeG DNA vaccinated groups. For FaeG-specific IgA, lower numbers of ASC could only be detected in the Cerv LN of most animals in all vaccinated groups (0–29 ASC/5 × 10⁶ MC).

In the pWRGFaeGopt group, the oral F4 boost induced comparable FaeG-specific antibody responses in the Cerv LN and the spleen as the third i.d. DNA immunization. Indeed, similar mean numbers of FaeG-specific IgG ASC were detected in the Cerv LN of orally and i.d. boosted pigs (122 and 131 ASC/5 × 10⁶ MC, respectively). In addition, also mainly FaeG-specific IgM ASC were found in the spleen of the orally boosted pigs of this group. In the pWRGFaeGopt + LT and the pWRGFaeGopt + vitD₃ group however, the oral F4 seemed to “boost” the systemic antibody response stronger than the third i.d. DNA immunization did. Indeed, in the Cerv LN of these groups, larger numbers of FaeG-specific IgG ASC were detected in the orally boosted pigs (143–696 and 69–854 ASC/5 × 10⁶ MC for the pWRGFaeGopt + LT and the pWRGFaeGopt + vitD₃ group, respectively) than in pigs that were immunized three times with DNA. Moreover, the FaeG-specific ASC in the spleen of the orally boosted pigs were mainly IgG (39–96 and 28–53 ASC/5 × 10⁶ MC for the pWRGFaeGopt + LT and the pWRGFaeGopt + vitD₃ group, respectively), whereas it was mainly IgM following the third DNA immunization. After the oral F4 boost, FaeG-specific IgA ASC could not be detected in the Cerv LN. However, few FaeG-specific IgA ASC could be detected in the spleen of all three FaeG DNA primed groups (0–4 ASC/5 × 10⁶ MC, respectively).

In the GALT (Fig. 5), low numbers of IgA and IgM ASC could be detected in the orally boosted pigs of the

pWRG7079 control group (up to 3 and 12 ASC/5 × 10⁶ MC, respectively), whereas higher numbers were seen in some FaeG DNA primed orally boosted pigs. Indeed, high numbers of IgM ASC could be detected in the IPP, JPP and Mes LN of one F4R-positive pig primed with pWRGFaeGopt + vitD₃ (10–173 ASC/5 × 10⁶ MC). An improved intestinal IgM response by DNA priming could also be observed in the LP of two F4R-negative pigs of the pWRGFaeGopt + LT group (21 and 35 ASC/5 × 10⁶ MC), and in the IPP and JPP of one of these pigs and of one pig of the pWRGFaeGopt group (up to 31 and 28 ASC/5 × 10⁶ MC, respectively). For these pigs, the numbers of IgA ASC were between 0 and 7 ASC/5 × 10⁶ MC in the IPP, JPP and Mes LN and up to 25 ASC/5 × 10⁶ MC in the LP.

The oral F4 boost seemed to weakly induce an IgG response in the GALT of DNA primed pigs. Indeed, IgG ASC could be detected in the GALT of some of the FaeG DNA primed pigs, whereas none of the control pigs showed IgG ASC in their GALT (Fig. 5). However, only in the F4R-positive pig of the pWRGFaeGopt + vitD₃ group with high numbers of intestinal IgM ASC, the numbers of FaeG-specific IgG ASC were consistently high in all examined GALT (22–346 ASC/5 × 10⁶ MC). In all other DNA primed pigs which received an oral F4 boost, except for one pig of the pWRGFaeGopt group, the numbers of IgG ASC were between 1 and 14 in the Mes LN. This was irrespective of the F4R status of the animals and occurred in higher numbers in both adjuvanted groups. In the other GALT, FaeG-specific IgG ASC could be detected in low numbers (1–4 ASC/5 × 10⁶ MC) (Fig. 5). This was mainly seen in the pWRGFaeGopt + LT group and more sporadically in both other DNA primed groups.

After three i.d. DNA immunizations, none of the pigs showed a clear FaeG-specific IgG, IgA or IgM response in their GALT (data not shown).

3.2. Experiment 2: Cellular responses following DNA vaccination

3.2.1. Cytokine expression profiles in the local draining lymph node

To assay cytokine mRNA expression profiles following DNA vaccination, induction of IL-1α, IL-6, TNF-α (pro-inflammatory cytokines), IL-2, IFN-γ (Th1-like cytokines), IL-4, IL-10 (Th2-like cytokines) and TGF-β (Th3-like cytokine) mRNA expression was evaluated 24 and 48 h psi (Fig. 6, Table 2).

At 24 h psi, a proinflammatory cytokine mRNA expression could already be observed after injection of the empty pWRG7079 vector for TNF-α, IL-1 and IL-6

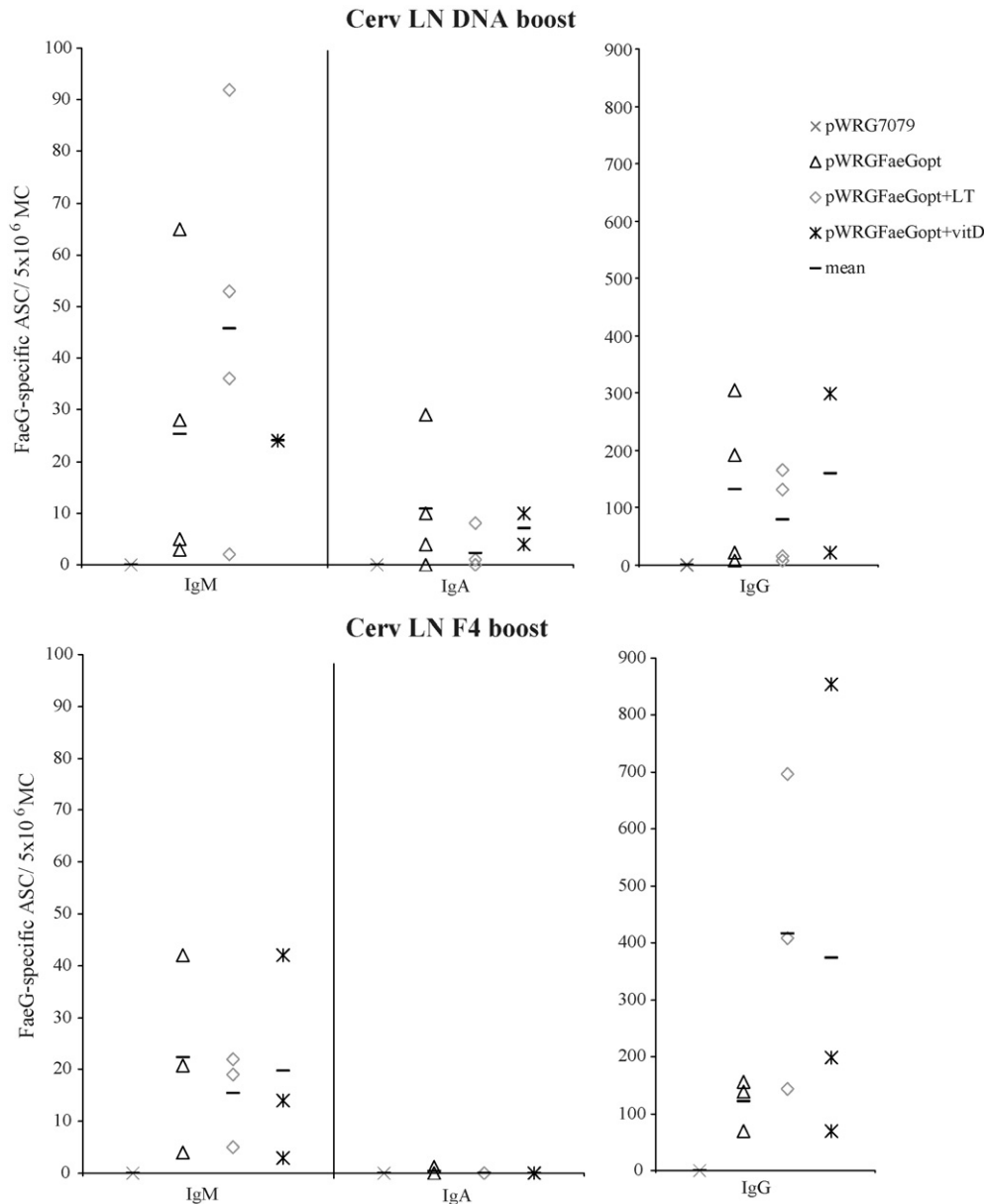


Fig. 3. FaeG-specific IgM, IgA and IgG ASC/ 5×10^6 MC in the Cerv LN 6 or 7 days (day 48 or 49 ppi) after the third i.d. DNA vaccination or the oral F4 boost. Pigs were immunized as shown in Table 1.

(1.6-, 3.2- and 9.4-fold, respectively) and to a lesser extend after PBS injection for IL-1 and IL-6 (1.5- and 6.7-fold increase, respectively). Only the IL-6 mRNA expression was increased more strongly by the FaeG expression (pWRGFaeGopt group, 18.3-fold). A similar increase in IL-6 mRNA expression could also be observed in the pWRGFaeGopt + vitD₃ group (14.5-fold), but not in the pWRGFaeGopt + LT group, as the mean increase in IL-6 mRNA expression in this group (3.7-fold) was even lower than in the control groups. At

48 h psi, the IL-6 mRNA expression was already strongly decreased, whereas the IL-1 mRNA expression was increased in the vitD₃ group. TNF- α mRNA expression remained similar in most groups.

For IL-10, IFN- γ and TGF- β mRNA, effects were most pronounced 48 h psi. Expression of TGF- β mRNA expression was increased following FaeG DNA vaccination and addition of LT and vitD₃ enhanced this increase. A strong increase in IL-10 mRNA expression could be observed for one pig of the pWRG7079, the

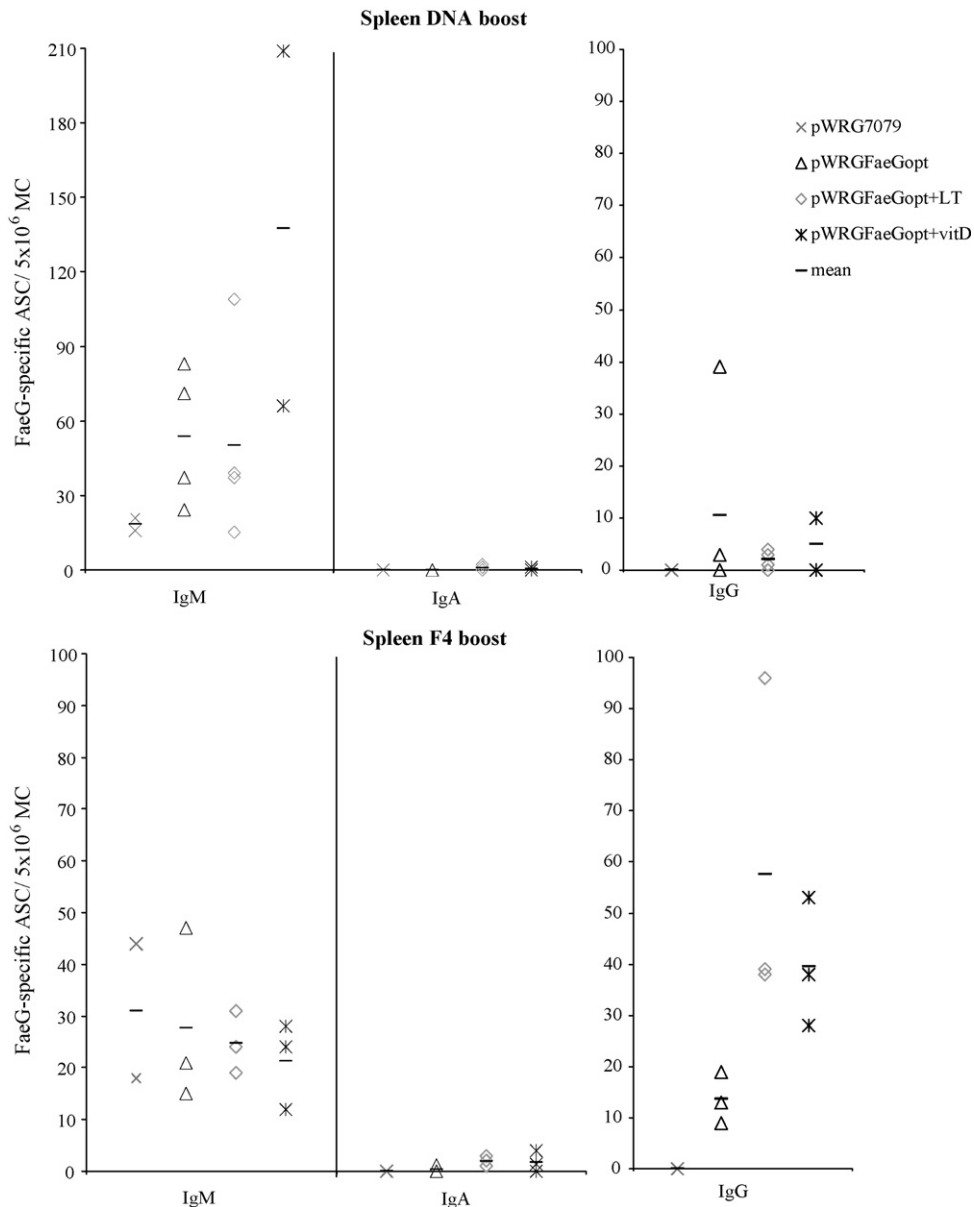


Fig. 4. FaeG-specific IgM, IgG and IgA ASC/ 5×10^6 MC in the spleen 6 or 7 days (day 48 or 49 ppi) after the third i.d. DNA vaccination or the oral F4 boost. Pigs were immunized as shown in Table 1.

pWRGFaeGopt and the pWRGFaeGopt + vitD₃ group, whereas none of the pigs from the LT adjuvanted groups showed this increase. For IFN- γ , a strong increase in mRNA expression could only be observed for one pig of each adjuvanted group.

IL-2 and IL-4 mRNA were hardly detected and are therefore not shown.

3.2.2. FaeG-specific lymphocyte proliferation

To analyze cellular (memory) immune responses following i.d. DNA vaccination, MC isolated from

the Cerv LN 48 h psi or from the spleen 6 or 7 days after the third immunization (pigs from experiment 1) were restimulated in vitro with F4 (Fig. 7).

At 48 h psi, a strong FaeG-specific proliferation could only be observed in MC from the Cerv LN of 3 on 4 pWRGFaeGopt + LT (cpm between 36,921 and 134,394) and 1 on 3 pWRGFaeGopt + vitD₃ vaccinated pigs (73,606 cpm). Interestingly, for each pig that showed a strong increase in IL-10 mRNA expression, the FaeG-specific proliferation was decreased more

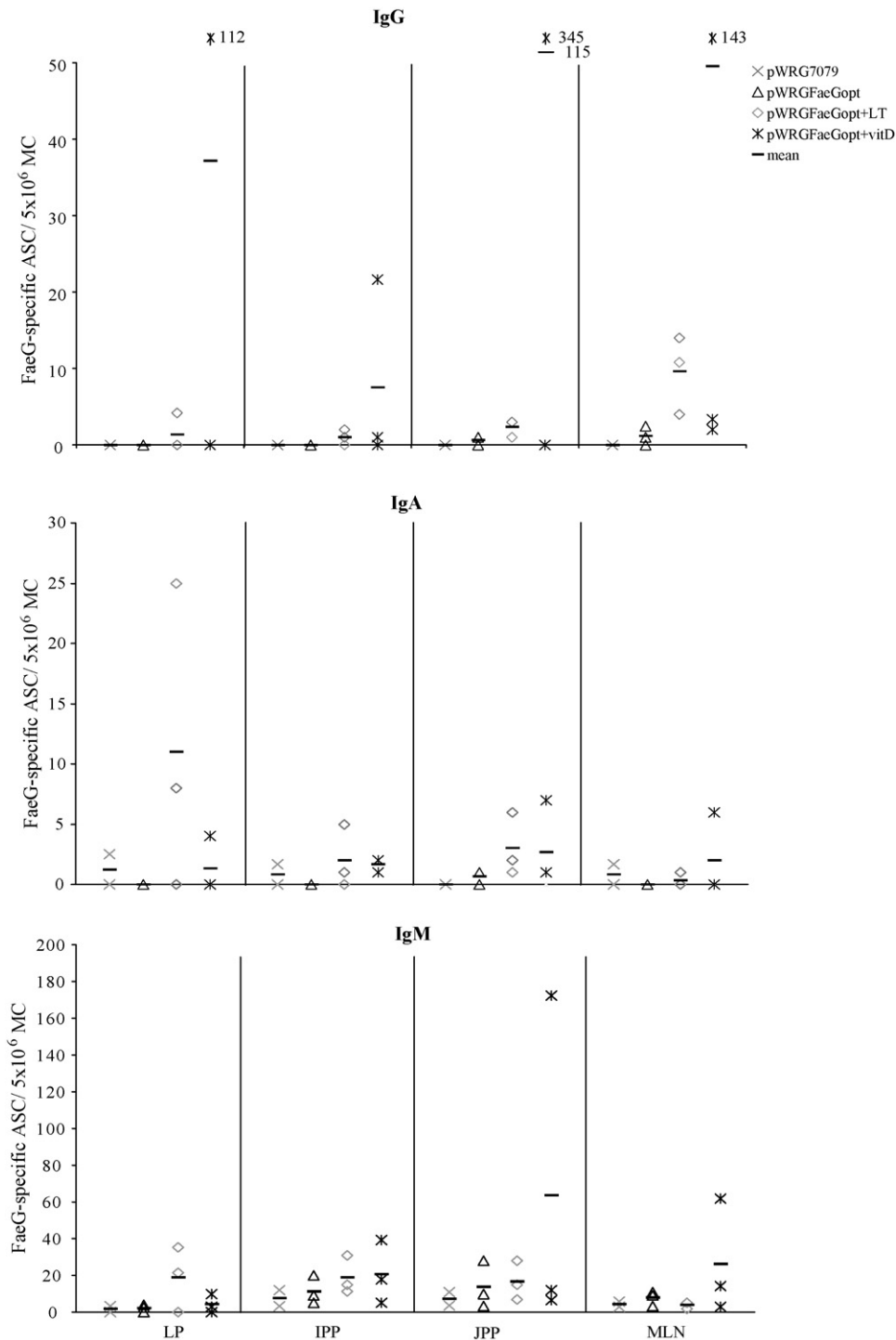


Fig. 5. FaeG-specific IgG, IgA or IgM ASC/5 × 10⁶ MC in the LP, IPP, JPP and MLN 6 or 7 days (day 48 or 49 ppi) after the oral F4 boost. Pigs were primed by 2 i.d. DNA immunizations as shown in Table 1.

than 1.7-fold compared to spontaneous medium proliferation (data not shown).

Also for spleen cells isolated after three DNA immunizations, FaeG-specific lymphocyte proliferation

was best induced in the pWRGFaeGopt + LT group. Indeed, a clear FaeG-specific proliferation (cpm > 5000) could be observed for 3 on 4 pigs of this group (cpm between 8420 and 23,167), compared

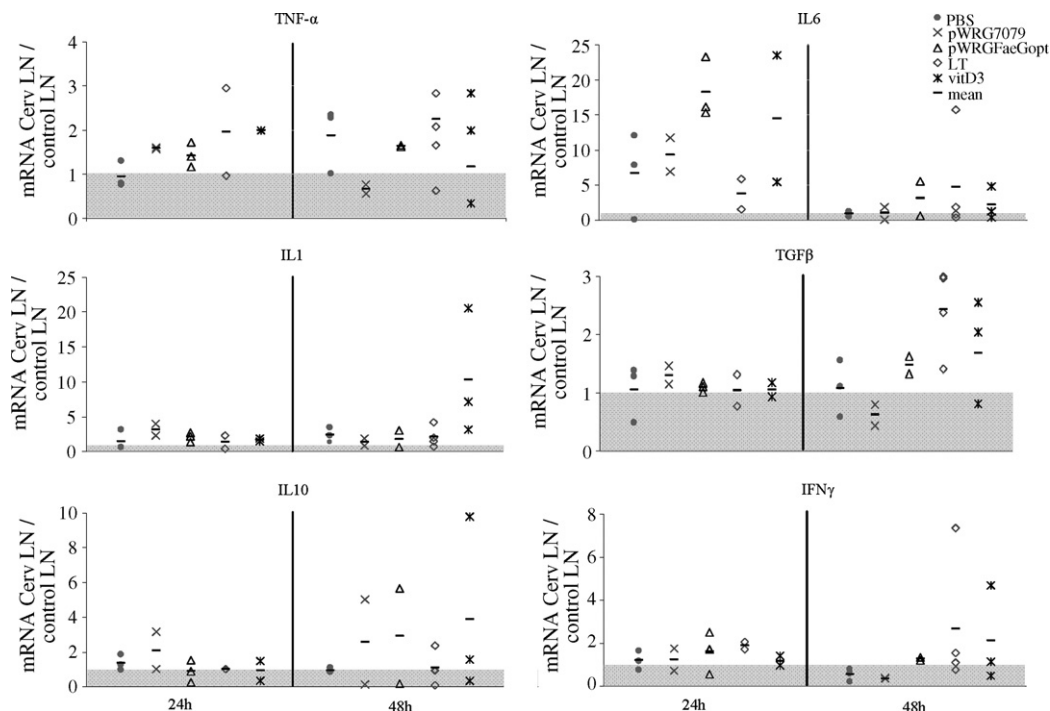


Fig. 6. Cytokine mRNA expression (TNF- α , IL-6, IL-1, TGF- β , IL-10 and IFN- γ) at 24 and 48 h psi. Pigs are immunized as shown in Table 2. Increases in cytokine mRNA expression by i.d. DNA vaccination are given as a ratio = (copy number of target cytokine in the Cerv LN/NF in the Cerv LN)/(copy number of target cytokine in the control Cerv LN/NF in the control Cerv LN).

to 1 on 4 pigs of the pWRGFaeGopt and 1 on 2 pigs of the pWRGFaeGopt + vitD₃ group (cpm of 7758 and 8254, respectively). Moreover, in both the Cerv LN after 2 DNA immunizations and the spleen after 3 i.d. DNA vaccinations, the mean cpm value was significantly higher in the pWRGFaeGopt + LT group than in the pWRGFaeGopt group. After the oral F4 boost, a clear FaeG-specific proliferation was only seen in 1 on 3 pigs of the pWRGFaeGopt + LT group (18,483 cpm).

Spontaneous proliferation was 2260 ± 431 for the Cerv LN and 1888 ± 213 for the spleen.

4. Discussion

In a first experiment, a heterologous i.d. pWRGFaeGopt DNA prime (2 \times)-oral F4 protein boost immunization and a homologous i.d. pWRGFaeGopt DNA vaccination (3 \times) were compared for their capacity to induce systemic and mucosal responses in pigs. Although i.d. DNA vaccination efficiently induced a serum IgG response, a clear FaeG-specific serum IgA or IgM response could not be observed in the present study. This was in contrast to our previous data where a

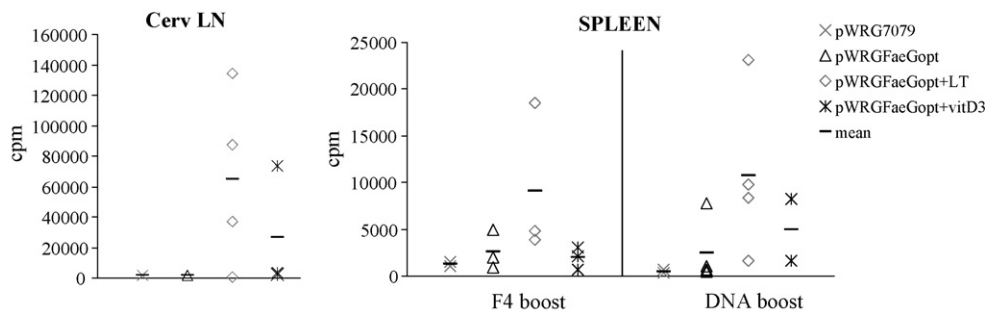


Fig. 7. FaeG-specific proliferation of Cerv LN MC at 48 h psi and spleen MC 6 or 7 days (day 48 or 49 ppi) after the third immunization. Pigs were immunized as shown in Tables 1 and 2, respectively. Significant differences are mentioned in the text.

low FaeG-specific IgA response could additionally be observed after i.d. pWRGFaeGopt immunization, but not when the LT vectors were added (Melkebeek et al., 2007). These contradicting findings might result from differences in origin of the pigs. Indeed, individual variation in immune responses, including Ig isotype responses, has been described for outbred pigs and has been attributed to differences in genetic background and environmental factors (Crawley et al., 2003; Wilkie and Mallard, 1999). A second reason could be an unnoticed minor contact with F4. Indeed, it has been described that intestinal priming can result in an IgA response following parenteral immunization (Svennerholm et al., 1980). Although farms were carefully selected for absence of F4⁺ ETEC related problems, litters were tested to be F4-seronegative and pigs were orally treated with colistine to prevent infection during the weaning period, a risk for such a contact can not absolutely be excluded.

Our inability to demonstrate a serum IgM response is likely due to the high cut off value in the IgM ELISA. An IgM background is often observed when young animals are tested. This is likely non-specific as even for gnotobiotic piglets, cut off values near 0.4 could be observed in F4-specific serum IgM ELISA (recent unpublished data from our lab). Furthermore, also in lymphoid tissues, a background of IgM ASC is often observed (Van den Broeck et al., 1999b; Verdonck et al., 2002; Snoeck et al., 2006a), even for F4R⁻ pigs which are resistant to F4⁺ ETEC infection.

After both the third i.d. DNA immunization and the oral F4 boost, antibody responses were mainly located systemically. After the i.d. DNA boost, responses in the adjuvanted groups were not better than those in the pWRGFaeGopt group, suggesting that other tissues, such as the bone marrow, might account for the higher serum IgG titer in these groups. Indeed, a study by Bianchi et al. (1999) demonstrated that the bone marrow is the major site of IgG production in pigs, containing 40% of all IgG secreting cells.

The presence of low numbers of IgA ASC in the Cerv LN following three intradermal DNA vaccinations is in line with our previous data indicating that i.d. DNA vaccination preceded by GM-CSF DNA injection primes a serum IgA response that can be boosted systemically (Melkebeek et al., 2006) but not mucosally (Melkebeek et al., 2007). An IgA response following systemic immunization was also seen in earlier studies in pigs (Van der Stede et al., 2002) and was more efficiently induced by immunizing in the neck compared to the back. The dorsal superficial cervical LN draining the neck region is connected to lymph

nodes draining mucosal sites (mandibular and medial retropharyngeal LN draining tonsils and nasal mucosa), which might be more beneficial for the induction of IgA (Vanderpooten et al., 1997).

Compared to the i.d. DNA boost, the oral F4 boost was clearly more immunogenic for the adjuvanted groups, as judged by an enhanced isotype switching towards IgG in the spleen and increased numbers of IgG ASC in the Cerv LN. This is in agreement with other studies demonstrating that prime-boost immunizations by alternating routes and/or immunization forms are as immunogenic or more immunogenic than homologous prime-boost immunizations (Baca-Estrada et al., 2000; McCluskie et al., 2002; Lauterslager et al., 2003). As observed previously (Melkebeek et al., 2007), boosting of systemic responses in parenterally primed pigs by oral F4 administration appeared to be independent of the F4R status of the pigs.

Besides inducing a systemic response, parenteral immunization, for example with live attenuated pathogens (Coffin et al., 1995) or in the presence of immunomodulating adjuvants like vitD₃, enterotoxins or chemical enhancers of cAMP (Enioutina et al., 1999, 2000; Van der Stede et al., 2004) has also been demonstrated to induce IgA ASC in the GALT. In our study however, an intestinal mucosal antibody response was nearly undetectable following i.d. DNA immunization. After the oral boost, the intestinal FaeG-specific IgA and IgM response were variable and an improvement by DNA priming could only be observed for a few pigs. In line with our previous results (Melkebeek et al., 2007), IgG ASC could be detected in the Mes LN of the DNA primed and especially of the adjuvanted groups. Moreover, although the presence of the F4R is required to induce IgA and IgM ASC in the Mes LN and other GALT of naïve pigs following multiple oral F4 administrations (Van den Broeck et al., 1999a), intestinal responses in DNA primed-orally boosted pigs were independent of the F4R status, suggesting an increased responsiveness of the parenterally primed animals to oral immunization. In contrast to other studies in mice and pigs demonstrating that vitD₃ enhances IgG but also IgA responses in the inductive sites of the GALT following parenteral immunization (Enioutina et al., 1999, 2000; Van der Stede et al., 2004), addition of vitD₃ to the FaeG DNA vaccine failed to prime for the intestinal mucosal IgA response that is desired to protect piglets against an F4⁺ ETEC infection.

In a second experiment, we evaluated the induction of cellular responses following i.d. DNA vaccination. A clear FaeG-specific lymphocyte proliferation could

only consistently be observed in the presence of the LT vectors. A strong positive influence of these vectors on the antigen-specific cellular response has previously been described (Arrington et al., 2002; Melkebeek et al., 2007). This might result from a higher phenotypic and functional maturation of APC (Bagley et al., 2002; Martin et al., 2002), resulting in a better antigen-presentation in the presence of LT and thus in a higher number of FaeG-specific memory lymphocytes and/or from a different subset of T(h)-cells and/or cytokine-profile after immunization. For the latter, evidence was obtained by analyzing cytokine mRNA profiles in the Cerv LN.

Pro-inflammatory cytokines were already weakly induced by the injection on itself (PBS group) and even slightly more by the DNA vector, with only the IL-6 mRNA expression being further up regulated by the FaeG expression. FaeG expression also resulted in an increased TGF- β mRNA expression, especially when an adjuvant was added. TGF- β has been suggested to be involved in the mucosal homing of immunocompetent cells (Enioutina et al., 1999) and is known to play a role in the isotype switching towards IgA (Lebman et al., 1990; Iwasato et al., 1994). For the expression of IL-10 (Th2-like) and IFN- γ (Th1-like) mRNA, a high variability could be observed among the animals. Furthermore, in line with other studies in pigs (Suradhat et al., 2003; Reddy et al., 2000), expression of IL-4 (Th2-like) and IL-2, could not be detected. It has been speculated that in pigs other molecules may substitute functionally for classical murine cytokines, like IL-4 and perhaps IL-2 (Reddy et al., 2000). IL-4 function might be substituted by IL-13 (Reddy et al., 2000), which has not been measured here. Consequently, we were not able to attribute a typical Th-cytokine profile to FaeG DNA vaccination and/or the LT vectors and vitamin D₃. What we did observe was a suppression of the FaeG-specific lymphocyte proliferation with increased IL-10 mRNA levels. This is in line with studies in man and cattle demonstrating that IL-10 inhibits Th1- and Th2-cell proliferation in these species (Del Prete et al., 1993; Brown et al., 1994). Moreover, although IL-10 mRNA expression was highly variable, the absence of a clear IL-10 response in the LT group might at least have contributed to the strong FaeG-specific proliferation observed in the presence of the LT vectors. Furthermore, IL-10 synergizes with TGF- β to increase the efficiency of IgA switching (Defrance et al., 1992), while IL-6, together with IL-5 acts to enhance the IgA secretion (Snoeck et al., 2006b). So, although no serum IgA response could be observed in the present study (as

discussed above), suppression of both IL-6 and IL-10 mRNA expression by the LT vectors might explain the suppressed serum IgA response in our previous study (Melkebeek et al., 2007).

In conclusion, even after the oral F4 boost, priming of an intestinal mucosal antibody response remained weak, varied among the pigs and seemed to require co-administration of the LT vectors or vitD₃. Moreover, even in presence of these adjuvants, we failed to demonstrate the secretory IgA response that is needed to be protective against ETEC. Therefore, further research should be directed towards enhancing the IgA response following DNA vaccination.

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